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Distribution and composition of the lysis cassette of *Lactococcus lactis* phages and functional analysis of bacteriophage ul36 holin

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Abstract

The bacteriophage lysis cassette, which comprises a lysin and a holin gene, was analyzed in 18 *Lactococcus lactis* phages. A muramidase motif was found in the lysins of c2-like phages, while an amidase motif was observed in the lysins of 936-like phages. Both amidase and muramidase types were detected among the P335 phages. The P335 lysins were separated into three groups based on amino acid sequence identity. A class I holin was recognized in 936-like and c2-like phages, whereas P335-like phages possess class II holins. The P335 holins were further divided into four groups based on sequence identity. Only the holins of 936-like phages contained putative dual-start motifs. The unusual lysis cassette of the highly virulent P335-like phage ul36 contains a unique holin (*orf74B*) upstream of a lysin which is present in several other P335-like phages. Using the λΔSthf system, we demonstrated that gpORF74B induces cell lysis at the same time as λΔSthf::S105, the effector of λ lysis. Transcriptional analysis of ul36 lysis cassette showed that first transcripts are detected 35 min after infection of *L. lactis* cells. The lysis clock of phage ul36 appears to be controlled by the late expression of the holin and lysin genes.
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1. Introduction

Large-scale fermentations of dairy products usually begin following the inoculation of milk with lactic acid bacteria such as *Lactococcus lactis*. These lactococcal cells are vulnerable to virulent bacteriophages and the ensuing cell lysis leads to lagging milk fermentations and low-quality fermented dairy products [1–3]. Due to their high prevalence in failed fermentations, only three lactococcal phage groups have been extensively studied, namely the c2, 936 and P335 species [3–6]. To date, the complete genomic sequences are available for twelve *L. lactis* phages, including two from the c2 species (bIL67 and c2), two from the 936 species (sk1, bIL170) and eight from the P335 quasi-species (bIL285, bIL286, bIL309, BK5-T, r1t, Tuc2009, TP901-1 and ul36) [7,8].

Typically, holin and endolysin are responsible for the cell burst after the intracellular phage development [9,10]. Holins are membrane proteins produced during the late stage of phage development, which are thought to accumulate in the cytoplasmic membrane as oligomers. These membrane-spanning proteins are responsible for the collapse of the membrane potential and the non-specific permeation of the cytoplasmic membrane, allowing the phage endolysin to degrade the host peptidoglycan. The holins are divided into at least two

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general classes based on the number of transmembrane (TM) domains found within the peptide sequence [9]. The class I includes proteins with three TM domains, while class II holins possess two TM domains.

Endolysins are divided into four groups based on their enzymatic activity: muramidase, transglycosylase, amidase, and peptidase [11]. However, only muramidases and amidases have been found in lactococcal phages [12–15]. Muramidases are responsible for the cleavage of the β1-4 *N*-acetylmuramic-acid-*N*-acetylglucosamine linkage in the peptidoglycan and they belong to the glycosidase enzyme class (E.C. 3.2.-). Amidases cleave the amide bond between *N*-acetylmuramoyl and L-amino acids in bacterial cell walls. The endolysin gene product also comprises at least two modules [16]. Often, the N-terminal part is associated with the enzymatic activity while the C-terminal section contains a substrate binding specificity.

The co-ordinated activity of both holin and endolysin eventually leads to the collapse of the cell wall and the release of phage progeny. Using the T7 inducible promoter system, it was determined that the holin and endolysin of lactococcal phages c2 and ϕLC3 (P335 species) were both needed for the lysis of *Escherichia coli* cells [10,12]. The controlled expression of an integrated lysis cassette via a transcriptional activator of phage ϕ31 (P335 species) was also used to develop an expression system that leaks β-galactosidase, but not peptidases in *L. lactis* [17,18]. Endolysins from c2-like phages were also exploited for accelerated cheese ripening (for a review see [19]).

Although both proteins are needed for cell burst, the timing of lysis is often controlled by the holin [9,10,20,21]. For example, the lysis of λ-infected *E. coli* cells depends on the 107 codons of the S gene, which encodes two proteins, S105 and S107 – the holin and holin inhibitor, respectively. The two peptides are produced as a result of two translation start points (dual-start motif). It is believed that optimal lysis will occur only when a sufficient level of S105 has accumulated in the membrane. The early lysis of the infected-cell is prevented by the S107 holin inhibitor. Although the lysis cassette of lactococcal phages appears to have the same gene organization as λ [12–15], little is known about the holins and the lysis clock of lactoccocal phages.

The virulent lactococcal phage ul36 belongs to the P335 species and its complete genome sequence was recently determined [8]. A unique putative lysis cassette was identified at the end of the late gene module and *orf74b* was ascribed as a putative class II holin gene with no dual-start motif and *orf429* as a putative endolysin gene coding for a member of the muramidase group. The aims of this study were to compare the lysis cassette composition of lactococcal phages and ascertain the holin activity of ORF74B of phage ul36 using the λΔSthf system and transcriptional analysis [22,23].

2. Materials and methods

2.1. Bacterial strain, phage and media

Lactococcus lactis SMQ-86 and SMQ-482, hosts for bacteriophage ul36, were grown at 30 °C in M17 [24] supplemented with 0.5% glucose. Phage ul36 [5] was propagated as described previously [25]. *E. coli* LE392 and derivate lysogens were grown at 30 °C with aeration for overnight cultures and pre-induction incubation as previously specified [23].

2.2. Sequence retrieval and analysis

The sequence analyses were performed using the Genetic Computer Group software package, version 10.0, including GenBank release 120.0, GenPept release 120.0, EMBL (Abridged) release 64.0, PIR-Protein release 66.0, NRL_3D release 27.0, SWISS-PROT release 36.0, SP-TREMBL release 15.0, PROSITE release 15.0. PSI-BLAST and Advanced Blast Search 2.1 were used for sequence comparisons with databases [26]. Membrane spanning motifs were determined using HMMTOP 2.0 (www.enzim.hu/hmmtop) [27] and MEMSAT (www.cs.ucl.ac.uk/staff/d.jones/memsat.html) [28]. Signal peptides were predicted using SignalP V2.0 (www.cbs.dtu.dk/services/SignalP-2.0) [29,30]. The complete genomic sequence of the lactococcal phage ul36 is available under the GenBank accession number AF349457 [8]. The putative holin gene corresponds to *orf74b* (coordinates 34854 to 35078) and the putative lysin gene to *orf429* (35075 to 36364). The following lactococcal phage sequences were used: sk1 (AF011378) [31], bIL170 (AF009630) [32], and ϕUS3 (M90423) [13] for the 936 species, c2 (L48605) [33], bIL67 (L33769) [34], and vML3 (X16178) [14] for the c2 species, r1t (U38906) [35], Tuc2009 (AF109874) [36], ϕLC3 (AF242738) [12], TP901-1 (AF304433) [37], bIL285 (AF323668), bIL286 (AF323669), bIL309 (AF323670) [38], TPW22 (AF066865) [39], BK5-T (AF176025) [40], ϕ31 (AJ292531) [41], and ϕAM2 (AF262017) for the P335 species. Two oenococcal phage sequences were also retrieved: fOg44 (AF047001) [42], and ϕ10MC (AF049087) [43].

2.3. Construction of λΔ*Sthf*::*ORF74B* and determination of lysis curves

The functional analysis of ul36 putative holin (ORF74B) was performed using the λΔSthf system detailed elsewhere [23]. The ul36 DNA encoding for *orf74b* was amplified by PCR using the synthetic primers FUL36 (5′-ATAC**GAATTC**ATGATTTTTAA CAAC AAGTTTTACAACG), and RUL36 (5′-ATAC**GAAT TC**TCATTTGTTTTCCTCCGTATCATTTGGC). The resulting amplicon was digested with *Eco*RI and ligated

to λΔSthf arms prepared as previously described [23]. The ligation mixture was packaged into λ particles using packagene system according to the manufacturer's instruction (Stratagene). Phage particles were then used for lysogenisation of *E. coli* LE392. Growth and lysis kinetics of λΔSthf::ORF74B were monitored using optical density at 600 nm (OD_{600nm}), and compared to curves of λΔSthf (negative control), as well as the λΔSthf::S105 and λΔSthf::S107 curves (positive controls) [23]. During the pre-induction phase, cultures were grown at 30 °C until the $OD_{600\ nm}$ reached 0.3. The induction was carried at 42 °C for 20 min, followed by a transfer to 37 °C for the remainder of the incubation period. Cultures were aerated during each experiment steps. The experiments were performed at least three times.

2.4. RNA manipulations

RNA of *L. lactis* cells infected by phage ul36 was isolated as described previously [44]. Slot blot was realized using the Bio-Dot SF apparatus (Bio-Rad) and performed as outlined elsewhere [45], using 1 μg RNA per slot. The probe corresponded to the coding regions of the holin and lysin genes and was made of a DIG-labeled PCR amplicon using the PCR DIG-labeling kit (Roche Diagnostics). Hybridization, wash and detection steps were performed as suggested by manufacturer (Roche Diagnostics). According to the ul36 sequence, the primers used were: hol1 5′-CAACGTCATCAAATGGGCTG (34,877–34,896) and lys2 5′-AGCCCCAGATTGACCG CCTG (36,201–36,220).

3. Results and discussion

3.1. Lysins of lactococcal phages

The composition of 18 lactococcal phage lysins was analyzed to predict the type of cell wall hydrolase activities. The list of lactococcal phages included three virulent c2-like phages (c2, bIL67, and vML3), three virulent 936-like phages (sk1, bIL70, and ϕUS3), and 12 members of the proposed P335 quasi-species (ϕ31, ϕAM2, bIL285, bIL286, bIL309, BK5-T, ϕLC3, r1t, Tuc2009, TP901-1, TPW22, and ul36). All the P335-like phages examined in this study are temperate phages except for ϕ31 and ul36 that are virulent. The *O*-glycosyl hydrolase motif pattern (pfam00959) was found in the three sequences available for c2-like phages (Table 1). The presence of this motif linked these lactococcal endolysins to other phage glycosyl hydrolase including λ lysosyme as well as an important component of the cell-puncturing device of phage T4 [46]. The conserved aspartic acid residue involved in the catalytic activity of the lysosyme was present in these three lactococcal phage lysins [43]. Conversely, an amidase motif (pfam01510) was observed in the three lysins available for 936-like phages (Table 1). This motif is also found in the lysin of phage T7 for which the structure was determined and showed to contain two conserved histidine residues involved in zinc binding [47].

Endolysins of both amidase and muramidase types were found among the twelve P335 phages (Table 1). These endolysins were separated in three groups based on a minimum of 60% identity between putative gene products (Fig. 1). The P335 endolysins of group I (ϕAM2, ϕLC3, TP901-1, Tuc2009, TPW22, and ul36) contain a glycosyl hydrolase motif (pfam01183.5) commonly found in muramidases. A putative peptidoglycan-binding domain (pfam01476) was also repeated twice at the C-termini of these endolysins. The P335 endolysins of group II (BK5-T, bIL285, bIL286, and bIL309, and ϕ31) possess the domain of the NLP/P60 family (pfam00877) of unknown function and a peptidoglycan-binding domain (pfam01471). These endolysins also have a weak homology with the N-terminal segment of the well-characterized *N*-acetylmuramoyl-L-alanine amidase of pneumococcal phage Dp-1. The third group contains only the endolysin of phage r1t. This endolysin

Table 1
Comparison of the lysis cassette of different lactococcal phage species

Species	Phages	Holin			Lysin	
		Class	Dual start	Identity (%)[a]	Specificity	Identity (%)[a]
c2-like phages	c2, bIL67, vML3	Class I	No	95	Muramidase	94–100
936-like phages	bIL170, sk1, ϕUS3	Class I	Yes	94–100	Amidase	78–79
P335 quasi-species		Class II	No			
I	ϕLC3, TP901-1, TPW22, Tuc2009			93–100	Muramidase	93–97[b]
II	bIL285, bIL286, bIL309, BK5-T			72–98	Amidase	96–100
III	ϕAM2, ul36			94	Same lysin as for module I	
IV	r1t			–	Amidase	–

[a] Amino acid identity percentage between genes of the members of each species.
[b] Including homologies with phage ul36 and ϕAM2 lysins.

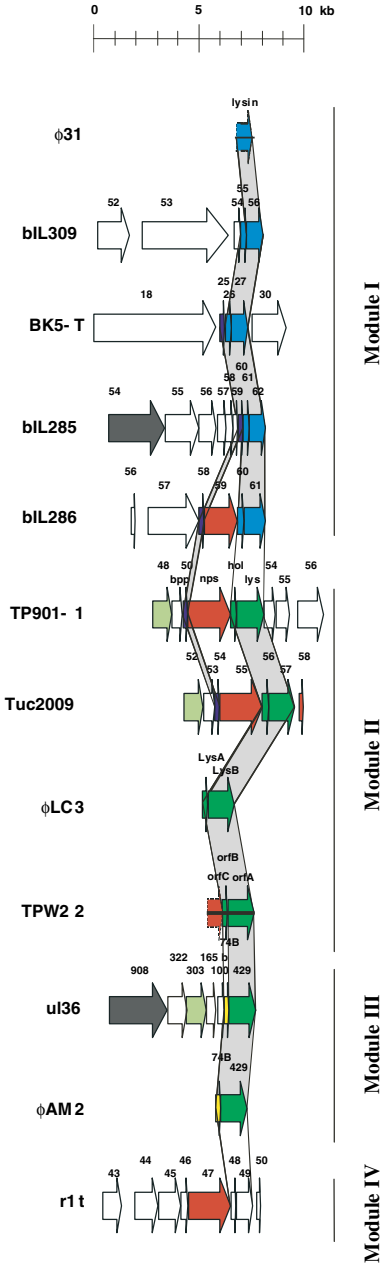

Fig. 1. Diversity of the lysis cassettes of P335-like phages. Proteins sharing at least 60% identity are represented using arrows with the same colors. Colorless arrows do not share any identity with other translated ORFs. Shaded boxes link homologous lysis cassettes and white boxes the non-homologous boxes.

carries a highly conserved amidase motif (pfam01510) in its N-terminal portion, as observed for the 936 species lysin. It did not however show any homology with other lactococcal endolysins.

3.2. Holins of lactococcal phages

The holins of 16 lactococcal phages were analyzed to predict the membrane-spanning patterns and potential dual-start motif at the N-termini. The holin genes of phage vML3 (c2) and ϕ31 (P335) were not available in the databases. The holins of the two phages of the c2 species contained three TM motifs, which is a feature of the class I holin [9]. No dual start motif was detected in these two holins. The holins of the three 936-like phages also belong to class I, but display a typical λ-like dual-start motif. The eleven P335 phages possessed a holin with two TM motifs and highly charged C-termini, which are characteristic of the class II holin [9]. No dual-start motif was found in these holins. However, as for their lysin counterpart, the holin sequences of the P335 phages are heterogeneous. The eleven holins from P335-like phages were divided into four groups based on a minimum of 60% identity between putative gene products (Fig. 1). The first group comprised holins from the phages ϕLC3, TP901-1, Tuc2009, and TPW22. The second group contained the holins of phages BK5-T, bIL285, bIL286, and bIL309. The putative holin of phage ul36 (ORF74B) was only homologous to the ϕAM2 holin (94% identity, 70/74 aa) and they constituted the third group. The holin of r1t was unique and represented a fourth group.

3.3. Composition of lactococcal phage lysis cassettes

Within the c2 and the 936 species, a holin gene was always linked to a specific lysin forming a homogeneous lysis module (Table 1). A different scenario was observed for P335-like phages, because four lysis modules were identified (Table 1). The heterogeneity of the P335 lysis cassettes is in agreement with the diversity observed in comparative genome analysis [8,38]. One temperate phage group (ϕLC3, TP901-1, Tuc2009, and TPW22) possesses a muramidase lysin and a class II holin with no dual-start motif. The second group comprises the temperate phages BK5-T, bIL285, bIL286, and bIL309, which have a lysin with a probable amidase activity and a distinct class II holin with no dual-start motif. The lysis cassette of the temperate phage r1t has the same general lysin and holin features as the ones found in the second group, but their deduced amino acid sequences are unique among the lactococcal phages analyzed in this study. Finally, the lysis cassette of the lactococcal virulent phage ul36 is similar to the temperate phage ϕAM2, which included a muramidase lysin similar to the first group, but coupled to a putative unique class II holin with no dual-start motif. These findings prompted us to analyze the probable holin function of ORF74B from ul36, a highly virulent phage lactococcal phage of the P335 species.

3.4. Experimental analysis of ORF74B from L. lactis bacteriophage ul36

The *E. coli* lambdaDeltaS genetic system (λΔSthf) was used to confirm the function of ORF74B as a phage

holin. This system was recently developed to enable the functional expression of holins from various phages in an isogenic λ background, and to carry out the qualitative evaluation of their ability to support lysis of *E. coli* cells [22,23]. Essentially, the lambda holin gene is replaced by a putative holin gene and the lysis curve of the chimeric phage is compared with the wild type [22]. The λΔSthf phage containing the holin gene (*orf74b*) of phage ul36 was obtained and named λΔSthf::ORF74B. The lysis curve of λΔSthf::ORF74B was established and compared to the antithetic action of phage λ S105 and S107 holin variants [23]. Twenty minutes after the induction of λΔSthf::ORF74B, the OD_{600nm} started to decrease, which is indicative of *E. coli* cell lysis (Fig. 2). A similar lysis time was observed with λΔSthf::S105 while lysis was significantly delayed with λΔSthf::S107. No lysis occurred with λΔSthf, the negative control. These results confirm that ORF74B acts as a holin in the *E. coli* background. It also suggests that no intrinsic lysis clock regulation is encoded by *orf74b*.

3.5. Transcriptional analysis of phage ul36 lysis module

To determine if the timing of lysis was caused by the late expression of the lysis module, the transcriptional analysis of phage ul36 was conducted by slot blot assay. RNA was isolated from *L. lactis* cells at time intervals during infection with phage ul36. The samples were hybridized with a 1.3-kb probe covering holin and lysin genes (AF349457, coordinates 34,877–36,220). Identical hybridization patterns were obtained with probes corresponding to the holin gene (34,877–35,070), and lysin gene (35,150–36,220) (data not shown). The transcripts were detected 35 min after the beginning of infection (Fig. 3). The delay in the expression of the lysis genes is consistent with the latent period and the high burst size previously reported for phage ul36 [44,48,49].

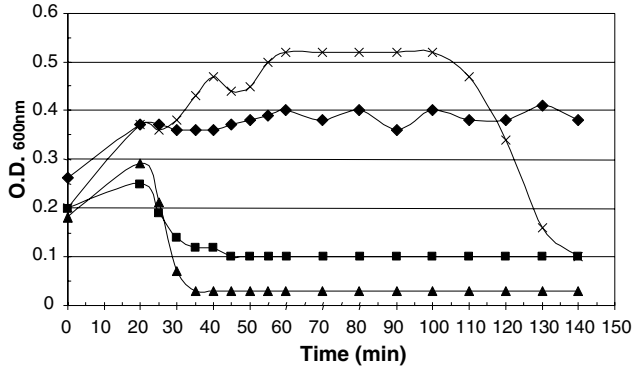

Fig. 2. Lysis curve of λΔSthf (◆) phage complemented with S105 (▲), S107 (×) or ORF74B of phage ul36 (■). The induction period (at 42 °C) corresponds to the time course between 0 and 20 min.

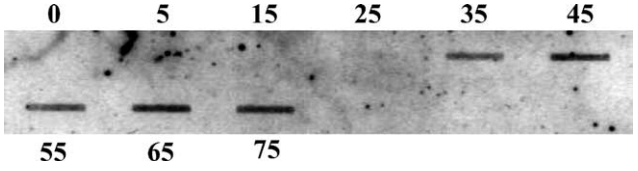

Fig. 3. Slot blot analysis of *L. lactis* cells infected with phage ul36. The slots contained 1 μg of total RNA isolated phage-infected cells after 0, 5, 15, 25, 35, 45, 55, 65 and 75 min. The DIG labeled probe covered both the holin and lysin genes.

3.6. Lactococcal phage lysis clock

The holin of 936-like phages carries a dual-start motif at the N-termini that may suggest the presence of an effector–inhibitor system as exemplified by the λ S-R lysis system [20]. For the oenococcal phage ϕ10MC and the lactococcal phage ϕvML3 (c2 species), the lysis clock was hypothetically attributed to the alternative initiation codon (TTG) of the lysin gene [42,43]. The resulting weak translation would be responsible for the cell lysis delay and the optimal progeny release. Alternative initiation codons are also likely for other c2-like phages (c2, bIL67), but not for members the 936- and P335 phage species. It has been postulated that the lysis clock of some P335-like phages may involve a signal peptide found in phages with a group II lysin (ϕAM2, ϕLC3, TP901-1, TPW22, Tuc2009, and ul36) [42]. However, the cell burst may still depend on the holin activity.

Complementation of λΔSthf convincingly demonstrated the lysis activity associated with the ORF74B of phage ul36. For both λΔSthf::ORF74B and λΔSthf::S105, cell lysis started 20 min after induction. Given the similar timing of cell lysis caused by S105 and ORF74B as well as the absence of a dual-start motif, it appears that holin activity of ORF74B is unregulated by intrinsic mechanism. The detection of the mRNA of the lysis module 35 min after phage infection suggests that the lysis clock of phage ul36 is regulated, at least in part, by the late expression of the holin and lysin genes. Since phage ul36 is a virulent phage that ruined milk fermentation [5], this delayed expression of the lysin and holin genes appears to be an effective lysis strategy.

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